

Novel Recognition Motif on Fibroblast Growth Factor Receptor Mediates Direct Association and Activation of SNT Adapter Proteins*

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Fibroblast growth factors (FGFs) stimulate tyrosine phosphorylation of a membrane-anchored adapter protein, FRS2/SNT-1, promoting its association with Shp-2 tyrosine phosphatase and upstream activators of Ras. Using the yeast two-hybrid protein-protein interaction assay, we show that FRS2/SNT-1 and a newly isolated SNT-2 protein directly bind to FGF receptor-1 (FGFR-1). A juxtamembrane segment of FGFR-1 and the phosphotyrosine-binding domain of SNTs are both necessary and sufficient for interaction in yeast and *in vitro*, and FGFR-mediated SNT tyrosine phosphorylation *in vivo* requires these segments of receptor and SNT. Our findings establish SNTs as direct protein links between FGFR-1 and multiple downstream pathways. The SNT binding motif of FGFR-1 is distinct from previously described phosphotyrosine-binding domain recognition motifs, lacking both tyrosine and asparagine residues.

Growth factors mediate biological responses through the coordination of multiple signals emanating from cell surface receptors. Receptors with intrinsic tyrosine kinase activity (RTKs)¹ may initiate several biochemical pathways by recruiting distinct cytosolic signaling proteins to specific receptor docking sites following receptor activation. RTK autophosphorylation can generate a set of receptor phosphotyrosine motifs that interact with Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains on target proteins, with the sequence surrounding each phosphotyrosine providing specificity for

particular SH2 or PTB domains (1, 2). For example, distinct phosphotyrosine motifs on epidermal growth factor receptor (EGFR) recruit phospholipase C- γ (PLC γ) and the adapter protein Grb-2 (3, 4). EGFR activates plasma membrane-associated Ras G proteins by co-recruitment of the Grb-2-associated Ras guanine nucleotide exchange factor Sos (5). The insulin receptor (IR) uses a variation on the above theme to trigger multiple signaling cascades. Activated IR recruits and tyrosine phosphorylates insulin receptor substrates (IRS-1, -2, -3, and -4) (6), which, in turn, serve as multifunctional adapter proteins by binding Grb-2/Sos, phosphatidylinositol-3'OH kinase, and Shp-2 tyrosine phosphatase (7–9).

Fibroblast growth factors (FGFs) stimulate receptors (FGFRs) that, similar to IR, do not directly recruit Grb-2/Sos or Shp-2. Nevertheless, several FGF-induced biological responses require the activation of both Ras and Shp-2 (10–13). Grb-2/Sos and Shp-2 bind to membrane-associated adapter proteins termed SNTs (14) following FGF-induced SNT tyrosine phosphorylation (15–17). A previously characterized myristoylated SNT protein, termed FRS2 (16), bears an N-terminal PTB domain and multiple sites of FGF-induced tyrosine phosphorylation, which engage the SH2 domains of Grb-2 and Shp-2 (16, 17).² Grb-2/Sos recruitment to SNT-1/FRS2 promotes Ras activation (16), whereas Shp-2 phosphatase may be activated by SNT engagement, analogous in mechanism to IRS-1-mediated Shp-2 activation (18). However, a direct interaction between activated FGFRs and SNT-1/FRS2 had not been established and seemed implausible because FGFRs lack NPx(pY) canonical PTB domain binding sites (19–21).

Using the yeast two-hybrid protein-protein interaction assay, we show here that SNT-1/FRS2 and a newly identified SNT-2 protein interact with FGFR-1. The PTB domain of SNTs and a juxtamembrane segment of FGFR-1 are both necessary and sufficient for this interaction, which is also seen between recombinant protein fragments *in vitro*, and these domains are also required for receptor-induced SNT tyrosine phosphorylation *in vivo*. These findings expand the repertoire of peptide motifs recognized by PTB domains and also establish SNT proteins as a family of FGF receptor signaling adapters that structurally and functionally resemble IRS proteins.

EXPERIMENTAL PROCEDURES

Reagents—The antibodies used were: monoclonal anti-EGFR for extracellular domain (Oncogene Science), 4G10 anti-phosphotyrosine, anti-PLC γ and anti-Shc (Upstate Biotechnology), anti-FGFR-1 C terminus and anti-GST (Santa Cruz Biotech), anti-Eps8 (Transduction Labs), 9E10 anti-myc tag (courtesy of T. Moran), peroxidase-conjugated secondary antibodies (Caltag). p13^{cas}-agarose was from Upstate Biotechnology Inc.

Human SNT cDNA Clones—Human SNT-1 cDNA (GenBankTM AF036717) encodes a 508-residue protein 96% identical to murine FRS2 (16), whereas human SNT-2 cDNA encodes a 492 residue protein bearing 49% sequence identity to SNT-1 (GenBankTM AF036718). Details of cloning procedures shall appear elsewhere.²

Expression of Epitope-tagged SNT Proteins in 3T3 Cells—SNT-1, SNT-2, and Shc cDNA open reading frames modified with a C-terminal triple myc epitope tag (22) were cloned in a SR α promoter-driven expression vector (23). Polymerase chain reaction with Pfu DNA polymerase (Stratagene) was used to engineer deleted forms of myc-tagged SNT-1: Δ PTB, residues 1–34/138–508; Δ M, 1–137/418–508; Δ C, 1–417. Vectors were introduced into NIH 3T3 cells or derivative clones carrying chimeric receptors by G418 or hygromycin selection of cotransfected

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[§] The abbreviations used are: RTK, receptor with intrinsic tyrosine kinase activity; SH2, Src homology-2; PTB, phosphotyrosine-binding; EGF, epidermal growth factor; EGFR, EGF receptor; PLC γ , phospholipase C- γ ; IR, insulin receptor; IRS, insulin receptor substrate; FGF, fibroblast growth factor; FGFR, FGF receptor; BD, binding domain; AD, activation domain; GST, glutathione S-transferase; WT, wild type; IP, immunoprecipitation; PY, phosphotyrosine.

² H. Xu and M. Goldfarb, manuscript in preparation.

plasmids. Selected cell pools treated for 10 min with 100 ng/ml acidic FGF, EGF, or mock were lysed in 1% Nonidet P-40, immunoprecipitated with 9E10, electrophoresed and Western blotted with 9E10 or 4G10, using peroxidase-conjugated secondary antibodies and ECL (Amersham Pharmacia Biotech) detection. Immunoprecipitates from cells incubated for 8 h with 100 μ Ci/ml [3 H]myristate (New England Nuclear) were electrophoresed, and labeled proteins were detected by fluorography.

Signaling by EGFR/FGFR Chimeras in 3T3 Cells—cDNAs for human EGFR (GenBank™ X00588) and murine FGFR-1 (GenBank™ U22324) were used to construct chimeric receptors, all of which bear the full extracellular domain of EGFR (residues 1–644): *E/FR1*, EGFR(1–644)/FGFR(377–822); *E/FR1-8*, EGFR(1–695)/FGFR(434–822); *E/FR1-10*, EGFR(1–644)/FGFR(377–762); *E/FR1-12*, EGFR(1–644)/FGFR(377–406, 434–822). pSR α vectors bearing chimeric receptor or native EGFR cDNA were cotransfected with pLTRneo into NIH 3T3 cells, and G418-resistant pools were lysed before or after 10 min of EGF treatment. p13^{suc1}-agarose captures and immunoprecipitates were electrophoresed and Western blotted with 4G10.

Yeast Two-hybrid Assays—Wild-type or mutant segments of FGFR-1 cDNA were cloned into pAS2-1 (CLONTECH) for expression as GAL4 DNA binding domain (BD) fusion proteins: BD-WT^(401–822), BD-K^(401–822/K514R), BD- Δ JM^(401–406, 434–822), BD-JM1^(401–434), BD-JM2^(401–451), BD (empty vector). Full-length or segments of SNT cDNAs were cloned into pACT-2 (CLONTECH) for expression as GAL4 activation domain (AD) fusion proteins: SNT-1^(2–508), SNT-1^(11–140), SNT-1^(164–508), SNT-2^(2–492), SNT-2^(11–141), AD (empty vector). The N-terminal SH2 domain (SH2-N) of rat PLC γ was also cloned into pACT-2. BD and AD plasmids were cotransformed into *Saccharomyces cerevisiae* CG1945 (*leu2, trp1, his3*, GAL₁UAS:*HIS3*, GAL₄UAS:*LACZ*) and selected for acquisition of plasmids on defined medium agar –Leu, –Trp, or for fusion protein interaction on –Leu, –Trp, –His, +1 mM 3-aminotriazole. Co-activation of *LACZ* was confirmed by colony lift X-gal staining. RIPA buffer cell lysates were immunoprecipitated with FGFR-1 antibodies, and Western blotted with 4G10- and BD-specific antibodies.

PTB Domain In Vitro Capture Assay—His₆-tagged FGFR-1^(401–451), FGFR-1^(763–822), or a random express sequence tag-derived 30-kDa protein fragment were expressed from pET3D vector in BL21DE3 and purified on nickel agarose. GST-SNT-1^(11–140) (PTB domain) and native GST were expressed from pGEX2T in *Escherichia coli* BL21DE3, purified on glutathione-agarose, and eluted with free glutathione. Nickel agarose conjugates (5 μ l) were used to capture aliquots of GST proteins in Nonidet P-40 lysis buffer, which were eluted, electrophoresed, and Western blotted with anti-GST. To detect His-tagged proteins, aliquots of proteins on nickel agarose conjugates were electrophoresed and stained with Coomassie Blue.

RESULTS AND DISCUSSION

Sequence Similarities between SNT and IRS Protein Families—SNTs were originally characterized as 85,000–90,000 apparent molecular weight proteins that undergo rapid NGF- and FGF-induced tyrosine phosphorylation in neuronal cells and that bind to immobilized yeast cell cycle protein p13^{suc1} (14). Tyrosine-phosphorylated p13^{suc1}-binding proteins were later characterized in several non-neuronal cell lines stimulated with FGF (15). We have cloned and characterized two human SNT genes encoding structurally related proteins. SNT-1 is 508 amino acids in length and is the human homolog of murine FRS2 (16), whereas SNT-2 is 492 residues in length and bears overall 49% amino acid sequence identity to SNT-1. SNT-1(FRS2) likely corresponds to previously described p13^{suc1}-binding proteins (14–16), whereas SNT-2 protein displays faster gel electrophoretic mobility (see below) and does not bind to p13^{suc1} (data not shown). Both SNTs are myristoylated by virtue of the conserved N-terminal modification consensus sequence MGSCCS (Fig. 1, A and B). Both proteins also bear highly related PTB domains (Fig. 1A) and share several tyrosine-containing motifs in the C-terminal two-thirds of each protein that govern Grb-2 and Shp-2 recruitment following SNT tyrosine phosphorylation (16).²

IRS proteins are distantly related to SNTs. An N-terminal pleckstrin homology domain promotes IRS membrane association (25), and the PTB domain near the N terminus, which

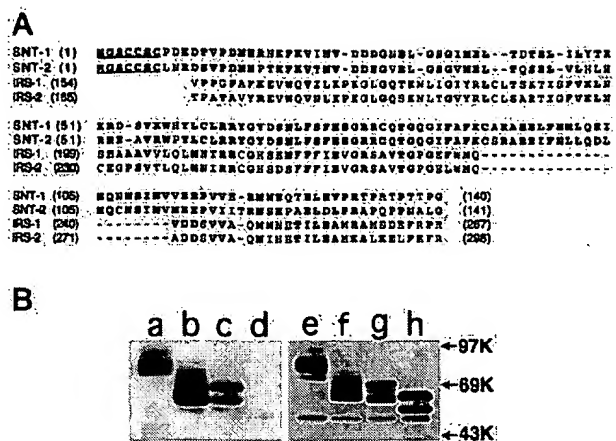


FIG. 1. SNT N-terminal sequence motifs. A, sequence alignments. SNTs have identical N-terminal myristoylation consensus motifs (underlined) and 60% identical PTB domains (residues 11–140 and 141) (identities and conservative substitutions in **boldface type**). IRS-1 and IRS-2 PTB domains (residues 154–267 and 185–298, respectively) are aligned, with residues related to either SNT in **boldface type**. B, SNT myristoylation. NIH 3T3 cells expressing myc-tagged SNT-1 (lanes a and e), SNT-1 Δ PTB (lanes b and f), SNT-2 (lanes c and g), or Shc (lanes d and h) protein were cultured with [3 H]myristate, and lysates were immunoprecipitated with 9E10 anti-myc monoclonal, followed by 7.5% polyacrylamide SDS gel electrophoresis and fluorography (lanes a–d). Unlabeled lysates were immunoprecipitated with 9E10, electrophoresed, and Western blotted with 9E10 to detect myc-tagged proteins (lanes e–h).

promotes IRS association with IR (20), is 20% identical to SNT PTB domains (Fig. 1A). The remainder of IRS proteins bear sites for IR-mediated tyrosine phosphorylation governing recruitment of IRS targets (7–9). Similarities between IRS and SNT proteins suggested that SNTs could serve as FGFR signaling adaptors analogous in function to IRS proteins.

The Juxtamembrane Segment of FGFR-1 Is Required for Ligand-induced Tyrosine Phosphorylation of SNTs—SNT proteins are tyrosine phosphorylated in response to FGF and neurotrophin receptor stimulation, but not following stimulation of other receptors, including IR and EGFR (14, 15). To localize FGFR domains required for SNT tyrosine phosphorylation, chimeric receptors were constructed bearing different segments of FGFR and EGFR transmembrane and cytoplasmic regions. All chimeras contained the EGFR extracellular domain, so that chimeric receptor signaling could be assayed in NIH 3T3 cells lacking endogenous EGFR (15). SNT was detected by capture with p13^{suc1}-agarose followed by anti-phosphotyrosine Western blot (14). As shown in Fig. 2A, EGF stimulation of E/FR1, which bears the entire transmembrane and cytoplasmic regions of FGFR1, induced strong SNT tyrosine phosphorylation, whereas stimulation of EGFR or chimeras E/FR1-12 and E/FR1-8, in which juxtamembrane residues 407–433 of FGFR1 were either deleted or replaced with EGFR sequence, respectively, induced very weak SNT phosphorylation. E/FR1-8 and E/FR1-12 still could undergo autophosphorylation and induce tyrosine phosphorylation of PLC γ (27), Shc (24), and Eps8 (26) (Fig. 2A). By contrast, the C-terminally truncated E/FR1-10 chimera, which lacks FGFR-1 residues 763–822, could induce SNT tyrosine phosphorylation (Fig. 2A) but not PLC γ phosphorylation as expected (27).

Following transfection of myc-tagged SNT-1 and SNT-2 into 3T3 cells, FGF stimulation of endogenous FGF receptors and EGF stimulation of E/FR1 could induce tyrosine phosphorylation of both SNTs, whereas activated E/FR1-12 could not efficiently tyrosine phosphorylate either SNT (Fig. 2B), although SNT-1 still underwent reduction in electrophoretic mobility,

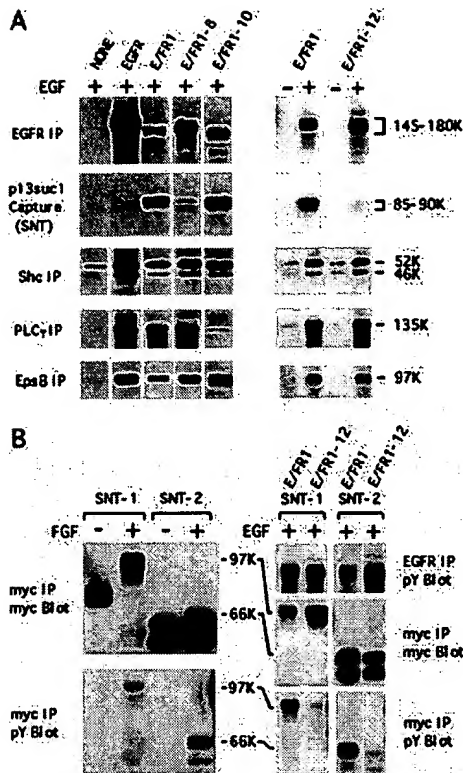


FIG. 2. FGFR-1 juxtamembrane sequence required for SNT tyrosine phosphorylation. *A*, signaling by chimeric receptors. 3T3 cells expressing EGFR or EGFR/FGFR chimeric receptors (E/FR1, E/FR1-8, E/FR1-10, or E/FR1-12) \pm EGF treatment (10 min) were lysed and subjected to anti-EGFR ectodomain IP, p13^{suc1} agarose capture (SNT), anti-Shc IP, anti-PLC γ IP, or anti-Eps8 IP. Proteins were electrophoresed and Western blotted with anti-phosphotyrosine (pY). *B*, SNT-1 and SNT-2 phosphorylation. 3T3 cells expressing myc-tagged SNT-1 or SNT-2 were treated \pm FGF, lysates were immunoprecipitated with anti-myc tag, electrophoresed, and blotted with anti-myc or anti-pY. 3T3 cells expressing E/FR1 or E/FR1-12, and myc-tagged SNT-1 or SNT-2 were treated with EGF, and lysates were analyzed for receptor tyrosine phosphorylation, myc-SNT expression, and myc SNT tyrosine phosphorylation (as above).

consistent with serine/threonine phosphorylation (15). The requirement of a FGFR juxtamembrane segment for SNT tyrosine phosphorylation suggested that this segment recognizes SNTs or intermediary proteins that link to SNTs.

The PTB Domain Is Required for SNT-1 Tyrosine Phosphorylation Following FGFR Stimulation—Myristoylation is required for efficient SNT-1/FRS2 tyrosine phosphorylation by anchoring the protein to the plasma membrane (16). To determine other SNT motifs required for FGF-induced tyrosine phosphorylation, myc-tagged SNT-1 proteins bearing deletions of residues 35–137 (Δ PTB), 138–417 (Δ M), or 418–508 (Δ C) were expressed in 3T3 cells and assayed for FGF-induced tyrosine phosphorylation. Although deletions of the middle or C-terminal portions of SNT-1 did not prevent FGF-induced tyrosine phosphorylation, deletion within the PTB domain completely abolished tyrosine phosphorylation (Fig. 3), despite retention of Δ PTB myristoylation (Fig. 1B) and association with the post-nuclear particulate fraction (data not shown), consistent with membrane localization. The requirement of the PTB domain for SNT tyrosine phosphorylation suggested that this domain interacts with FGFR or an intermediary protein.

Yeast Two-hybrid Assay Detects Direct Interaction between SNT PTB Domains and Receptor Juxtamembrane Motif—FGFR-1 and SNTs do not readily coimmunoprecipitate from mammalian cell lysates, suggesting the absence of stable

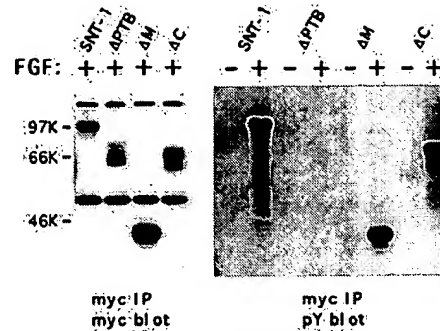


FIG. 3. PTB domain required for FGF-induced SNT-1 tyrosine phosphorylation. NIH 3T3 cells expressing myc-tagged full-length or truncated SNT-1 proteins were stimulated with FGF prior to lysis, and SNT proteins immunoprecipitated with 9E10 myc antibody were electrophoresed and Western blotted with pY or anti-myc.

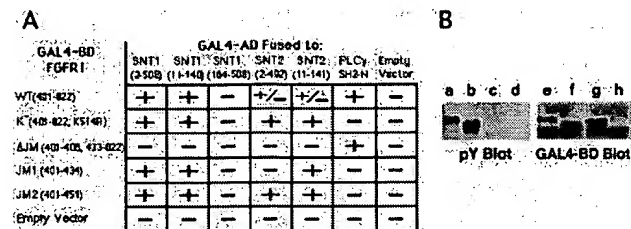


FIG. 4. Yeast two-hybrid assays detect SNT/FGFR interactions. *A*, table of interactions. Fragments of FGFR-1 cloned in pAS2-1 (GAL4 BD fusion) and of SNT-1, SNT-2, or PLC γ cloned in pACT2 (GAL4 AD fusion) were co-transformed into *S. cerevisiae* CG1945, which were plated either on -LT agar or -LTH + 1 mM 3-AT agar. +, (-LTH + 3-AT)/(-LT) colony ratios > 0.7 ; +/+, ratios 0.1–0.5; -, no colonies on -LTH + 3-AT. All colonies from + transformations were positive for β -galactosidase staining. *B*, tyrosine autophosphorylation of receptor fusion proteins. Anti-FGFR-1 immunoprecipitates from yeast expressing BD-WT (lanes a and e), BD- Δ JM (lanes b and f), BD-K⁻ (lanes c and g), and BD empty vector (lanes d and h) were electrophoresed and probed by Western blot with anti-pY (lanes a–d) or anti-GAL4 BD (lanes e–h).

FGFR/SNT complexes (15).³ We chose to use the yeast two-hybrid system to detect and characterize weaker FGFR/SNT interactions, as this assay has facilitated analysis of weak interactions between IR and IRS (28).

The cytoplasmic portion (residues 401–822) of wild-type (wt) or kinase-negative (K⁻) FGFR-1 were expressed as GAL4 DNA BD fusion proteins, whereas SNT proteins (residues 2 through the C termini) were expressed as GAL4 transcription AD fusion proteins, and yeast cotransformed with BD and AD fusion proteins were assayed for activation of GAL4-responsive HIS3 and LACZ reporter genes as evidence for direct protein-protein interaction. Both forms of FGFR-1 interacted with SNT-1 and SNT-2 (Fig. 4A), with kinase-deficient FGFR-1 BD vector giving a more robust response, probably reflecting a toxic effect of wild-type FGFR-1 expression in yeast. The ability of wild-type, but not kinase-negative, FGFR-1 BD fusion protein to undergo tyrosine autophosphorylation was shown by interaction of BD-WT with PLC γ SH2-N (Fig. 4A) and by anti-pY Western blot (Fig. 4B).

Deletion mutagenesis was used to map the interacting segments of SNTs and FGFR-1. SNT-1 and SNT-2 PTB domains (residues 11–140 and 11–141, respectively) interacted with wt and kinase-negative FGFR-1, whereas a large C-terminal segment of SNT-1 (residues 164–508) failed to interact with either receptor (Fig. 4A). Mutant BD- Δ JM, lacking residues 407–433 of FGFR-1 (identical to the deletion in E/FR1-12) failed to interact with PTB domain or full-length forms of either SNT

³ H. Xu and M. Goldfarb, unpublished data.



FIG. 5. Direct association of SNT-1 PTB and FGFR-1 juxtamembrane domains *in vitro*. A, FGFR-1-agarose capture of SNT-1 PTB domain. 50-ng aliquots of GST-SNT1⁽¹¹⁻¹⁴⁰⁾ (lanes b, d, f, and h) and native GST (lanes a, c, e, and g) proteins were electrophoresed directly (lanes a and b) or used for capture with agaroses bearing His-tagged proteins, followed by elution, electrophoresis, and detection by anti-GST Western blot. Captures with His-tagged FGFR-1⁽⁴⁰¹⁻⁴⁵¹⁾ (lanes c and d), FGFR-1⁽⁷⁶³⁻⁸²²⁾ (lanes e and f), or an unrelated 30-kDa protein (lanes g and h). B, Coomassie stain of agarose conjugates. Aliquots of nickel agaroses bearing His-tagged FGFR-1⁽⁴⁰¹⁻⁴⁵¹⁾ (lane a), FGFR-1⁽⁷⁶³⁻⁸²²⁾ (lane b), and 30-kDa protein (lane c) were electrophoresed on a 15% polyacrylamide SDS gel, which was Coomassie Blue-stained. His-tagged proteins are marked by arrows.

(Fig. 4A), while retaining the ability to undergo tyrosine autophosphorylation (Fig. 4B) and to interact with PLC γ SH2-N domain (Fig. 4A). BD-JM1, containing only FGFR-1 residues 401–434, interacted with both full-length SNT-1 and the PTB domains of both SNTs, demonstrating that residues 401–434 are both necessary and sufficient for interaction with SNT PTB domains. This region of FGFR-1 lacks asparagine and tyrosine residues found in other PTB domain recognition motifs.

Although SNT-2 PTB domain interacted with juxtamembrane FGFR-1 residues 401–434, full-length SNT-2 scored negative against BD-JM1 but did interact with a somewhat larger FGFR-1 segment (401–451) in BD-JM2 (Fig. 4A). This paradoxical observation may be a reflection of additional, uncharacterized contacts between FGFR-1 and SNT-2 proteins.

In Vitro Binding of Recombinant FGFR-1 and SNT-1 Protein Fragments—As an additional assay for confirming direct SNT/FGFR interaction, prokaryotically expressed fragments of FGFR-1 and SNT-1 were tested for their ability to associate *in vitro*. FGFR-1 juxtamembrane residues 401–451, and control peptides were expressed as polyhistidine-tagged proteins and purified on nickel-agarose beads, which were then used for *in vitro* capture of soluble GST-SNT1⁽¹¹⁻¹⁴⁰⁾ PTB fusion protein or native GST. Whereas agaroses bearing FGFR-1⁽⁷⁶³⁻⁸²²⁾ or an unrelated 30-kDa polyhistidine-tagged protein nonspecifically captured a small fraction of the input GST-SNT1⁽¹¹⁻¹⁴⁰⁾ protein, agarose bearing a juxtamembrane fragment of FGFR-1⁽⁴⁰¹⁻⁴⁵¹⁾ quantitatively captured GST-SNT1⁽¹¹⁻¹⁴⁰⁾ protein from solution but did not react with native GST (Fig. 5).

Conclusions—We have shown that a direct interaction between FGFR-1 and SNTs is required for receptor-induced SNT tyrosine phosphorylation. These findings establish SNTs as adapters that directly link activated FGF receptors to multiple downstream signaling pathways (Grb2/Sos and Shp-2), similar to the role of IRS proteins in governing insulin receptor signaling. The juxtamembrane sequences of FGFRs are not significantly related to cytoplasmic sequences in other RTKs, probably accounting for the failure of most RTKs to activate SNTs (14, 15).

PTB domains generally recognize peptide motifs containing the sequence NPx(pY) (19–21), although variant motifs for some PTB domains lacking phosphotyrosine or asparagine have been described (29–31). The SNT PTB domains bind to a region of FGF receptor lacking both tyrosine and asparagine

residues, thereby further expanding the repertoire of motifs recognized by PTB domains. The phosphotyrosine independence of SNT/FGFR-1 interaction suggests that SNTs may stably or transiently contact FGFR-1 prior to receptor activation.

A potentially important feature of SNT PTB domains is their retention of signature arginine residues at SNT positions 63 and 78 (Fig. 1A). The corresponding PTB domain arginines at positions 212 and 227 of IRS-1 interact with phosphotyrosine on insulin and interleukin-4 receptors (20, 21). We speculate that an alternative phosphotyrosine-dependent mode of epitope recognition may govern association of SNTs with other binding partners, such as the neurotrophin receptors.

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